Our Reference No. 9369-98

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	
Maurice Moloney, Joenel Alcantara and, Gijs van Rooijen	
	Group No.: 1656
Serial No. **09/402,488**	
	Examiner: David J. Steadman
Filed: **February 16, 2000**	
For: **Method For Cleavage of Fusion Proteins**	

DECLARATION UNDER 37 C.F.R. 1.132

Honourable Assistant Commissioner
For Patents
Washington, D.C. 20231

Sir:

I, Maurice Moloney, citizen of Ireland and resident of Calgary, Alberta, Canada, hereby declare and state as follows:

1. I am one of the named inventors of the above-referenced patent application (hereinafter "the application").

2. I am the Chief Scientific Officer at SemBioSys Genetics Inc., the owner of the application. I have been involved in research relating to plant molecular biology for over 20 years. I attach a copy of my curriculum vitae as Exhibit A.

3. I have reviewed the Official Action issued on December 30, 2005, in connection with the application. In particular, I note the rejection of claims 1, 4, 6-9, 13, 15 and 19 under 35 USC §103(a), claim 5 under 35 USC §103(a), claims 10, 16 and 48-49 under 35 USC §103(a) and claims 14 and 50 under 35 USC §103(a).

4. Pages 10-15 of the Office Action discuss the teachings of Ward *et al.* (US Patent 6,265,204), Walsh *et al.*, (J. Biotech 45:235-241) and Yonezawa *et al.* (Int J Pept Protein Res 47:56-61). At page 11, the Office Action cites these references in support of the position that:

> one of ordinary skill in the art would have recognized that chymosin is an appropriate endoproteinase for cleaving a fusion protein at a Phe-Met junction of a desired protein with an N-terminal chymosin pro-peptide.

At pages 14-15, the Office Action notes (i) Walsh's teaching of "a bovine kappa-casein chymosin cleavage site (cleavage between Phe-Met, wherein Phe is at the P1 position and Met is at the P1' position) as a cleavable linker in a fusion protein," (ii) knowledge in the art that "pro-peptides of chymosin from mammalian and fungal sources also have a Phe at the P1 position," and (iii) knowledge that "most" heterologous proteins have "a Met at the N-terminus," and alleges that a skilled artisan would be motivated to use a chymosin pro-peptide as a fusion protein linker as claimed in the application, because such a fusion protein would have a Phe-Met junction that allegedly would be specifically cleaved by chymosin. Those assertions are not supported by the cited references, however, and contradict knowledge in the art regarding the activity of chymosin.

5. I have reviewed the Ward, Walsh and Yonezawa references cited in the Office Action. I have also reviewed Visser *et al.*, Biochim Biophys Acta 438: 265-72 (1976), which is cited by Walsh, and Schattenkerk *et al.*, Recl. Trav. Chim. Pays-Bas 90: 1320-22, (1970), which is cited by Visser. These references (attached as Exhibits B and C)

contradict the assertion that chymosin can be used to cleave a fusion protein at any Phe-Met junction.

6. These references do teach that chymosin cleaves the substrate κ-casein at a Phe-Met bond, but they also teach that the primary structure of the amino acids surrounding the Phe-Met bond is essential to the cleavage reaction. For example, Walsh *et al.* teaches at page 239, second column, that "[s]pecific hydrolysis of Phe_{105}-Met_{106} of κ-casein at pH 6.8 by chymosin is dependent upon the composition and sequence of amino acid residues in an extended region of the primary structure." (Emphasis added.) Consistent with Walsh, the abstract and page 271 of Visser *et al.* teach that "the sequence -Ser-Phe-Met-Ala- with a further residue added to either end is necessary to induce any cleavage by the enzyme." Figure 1 (page 1321) of Schattenkerk *et al.* provides further data on this point, showing that methyl esters (used for their solubility) of the following κ-casein-based peptides were completely resistant to cleavage by chymosin (referred to as "rennin" in the paper):

Phe-Met

Phe-Met-Ala

Phe-Met-Ala-Ile

Phe-Met-Ala-Ile-Pro-Pro-Lys-Lys

Ser-Phe-Met

Ser-Phe-Met-Ala

Leu-Ser-Phe-Met

Schattenkerk also reports that the peptide Leu-Phe-Met-Ala was completely resistant to cleavage by chymosin. On the other hand, Schattenkerk teaches that methyl esters of other κ-casein-based peptides (*e.g.*, Leu-Ser-Phe-Met-Ala-Ile) exhibited 99% cleavage by chymosin. As summarized by Visser *et al.* on page 271, second paragraph, "it is apparent that a minimum chain length of five amino acids residues including the sequence -Ser-Phe-Met-Ala- is essential to bring about any cleavage of the Phe-Met bond."

7. These teachings contradict the assertion in the Office Action that "chymosin is an appropriate endoproteinase for cleaving a fusion protein at a Phe-Met junction of a desired protein with an N-terminal chymosin pro-peptide," because they show that chymosin does not cleave substrate proteins at any and all Phe-Met junctions.

8. I also draw the attention of the Examiner to the Examples provided in the current application (USSN 09/402,288). As seen in Example 1, and specifically in Figure 1, the deduced amino acid sequence of the GST-Pro-Hirudin fusion protein includes a Phe-Met bond between amino acids 153 and 154 that is not cleaved during the cleavage reaction. This is consistent with the findings of Visser *et al.* and Walsh *et al.* which demonstrate the need for additional amino acids in order for the Phe-Met bond to be a substrate for chymosin. In addition, the cleavage site of the fusion protein in Figure 1 is not between a Phe-Met bond, but is between a Phe-Val bond between amino acid residues 278 and 279. Likewise, the cleavage site of the His-Pro-cGH fusion protein in Figure 2 is not between a Phe-Met bond, but is between a Phe-Ser bond between amino acid residues 84 and 85.

9. In summary, while the prior art teaches that chymosin cleaves the substrate κ-casein at a Phe-Met bond, the prior art and the examples in the application demonstrate that chymosin does not cleave substrate proteins at any and all Phe-Met bonds that are present. Thus, the basis for the obviousness rejections set forth in the Office Action is contradicted by the art of record and the additional prior art references discussed above.

10. At pages 10-11, the Office Action states that "Methionine is usually the first amino acid of a given polypeptide." This assertion is not consistent with the knowledge in the art regarding the primary structure of polypeptides. While the methionine codon is the "start" codon, a vast number of functional proteins do not have methionine at

their N-terminus because they are derived *in vivo* from fusions with signal sequences, transit peptides and other labile linkers and undergo modifications which eliminate the N-terminal methionine. Thus, a recombinant polypeptide of interest will not necessarily have an N-terminal methionine, and a prochymosin-polypeptide junction will not necessarily have a Phe-Met sequence.

11. I also point out that invention described and claimed in the application would not have been expected from the references discussed above, because they specifically teach that the sequence -Ser-Phe-Met-Ala- is essential for the reported chymosin cleavage, and do not indicate that chymosin can cleave a substrate protein at a Phe-Val or Phe-Ser bond, as shown in the examples of the application.

12. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and, further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such a willful false statement may jeopardize the validity of the application or any patent issuing thereon.

April 7, 2006
Date

Maurice Moloney

Exhibit A

Dr. Maurice Moloney

SemBioSys Genetics Inc
110, 2985-23rd Avenue NE
Calgary, Alberta T1Y 7L3
(403) 250-5424 x758 Fax: (403) 250-3886
Email: moloneym@sembiosys.com

Dr. Maurice Moloney is the scientific founder of SemBioSys and serves as the company's chief scientific officer. He is also a professor in the Department of Biological Sciences at the University of Calgary and holds the Natural Sciences and Engineering Research Council of Canada (NSERC) Industrial Research Chair in plant biotechnology. Dr. Moloney's career in plant technology spans 20 years. Prior to founding SemBioSys, he spent seven years in his post at the University of Calgary, pursuing research on seed-specific gene expression, herbicide resistance and the plant cell cycle. Previously, he was the head of the Cell Biology Group at Calgene Inc., where he developed the first transgenic oilseed plants using Canola as the model. This resulted in a landmark patent in plant biotechnology and eventually became the basis of RoundUp Ready and Liberty Link Canola.

Dr. Moloney has published more than 70 original research papers and is an inventor on 22 issued or pending patent families. He serves on the advisory board of the National Research Council's Plant Biotechnology Institute and two other biotechnology companies. He serves on numerous Federal government committees including NSERC Council (the governing body of NSERC), CFI, National Research Council (Canada) and the Networks of Centres of Excellence programs. He was the co-president of the International Society for Plant Molecular Biology (ISPMB) Congress in 2000 and serves on the ISPMB board. Dr. Moloney has received a number of prestigious awards, including the Alberta Science and Technology (ASTECH) Award for leadership in Alberta Technology. He holds a Bachelor of Science degree in organic chemistry from Imperial College, University of London, and was awarded his doctorate in plant biochemistry from De Montfort University/Leicester Polytechnic in the United Kingdom.

Academic, Research and Industrial Appointments:

2004	Doctor of Science (*honoris causa*) University of Lethbridge
2003 – present	Member, Executive Committee of NSERC
2003 - present	Chair, Committee on Research Partnerships - NSERC
2002 - present	Appointed to the Natural Sciences and Engineering Research Council of Canada (NSERC) for a three year term
1999	ASTECH Award for leadership in Alberta Technology.
1995 – present	Professor, NSERC/Dow AgroSciences Industrial Research Chair of Plant Biotechnology, University of Calgary, Dept. of Biological Sciences
1994 – present	Founder and Chief Scientific Officer, SemBioSys Genetics Inc.
1990 – 1995	Associate Professor, University of Calgary, Dept. of Biological Sciences
1986 – 1990	Assistant Professor, University of Calgary, Dept. of Biological Sciences
1983 – 1986	Principal Scientist and Coordinator, Calgene Inc., Cell Biology Group
1979 – 1983	Royal Society European Postdoctoral Fellow, University of Lausanne, Institut de Physiologie et de Biologie Vegetales

1976 – 1979 Research Assistant, Leicester Polytechnic

Education:

1979 Doctorate in Plant Physiology, Leicester Polytechnic
1974 Bachelor of Science (Chemistry), Imperial College, University of London, UK

Research Contributions:

Articles in refereed publications (since 1995):

Abell, B.M., Hahn, M., Holbrook, L.A., **Moloney, M.M.** (2004) Membrane Topology and Sequence Requirements for Oil Body Targeting of Oleosin. The Plant Journal. 37: 461-70

Kühnel B, Alcantara, J., Boothe, J., van Rooijen, G., **Moloney, M.M.** (2003) Precise and Efficient Cleavage of Recombinant Fusion Proteins Using Mammalian Aspartic Proteases. Protein Engineering. Protein Engineering, Vol. 16 (10) pp. 777-783.

Seon, J.H., Szarka, S.J.., **Moloney, M.M.** (2002) A Unique Strategy for Recovering Recombinant Proteins from Molecular Farming: Affinity Capture on Engineered Oilbodies Journal of Plant Biotechnology 4(3) pp. 95 – 101.

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Nykiforuk, C.L., Furukawa-Stoffer, T.L., Huff, P.W., Sarna, M., Laroche A., **Moloney M.M.,** Weselake, R.J. (2002) Characterization of cDNAs Encoding Diacylglycerol Acyltransferase from Cultures of *Brassica napus* and Sucrose-Mediated Induction of Enzyme Biosynthesis. Biochimica et Biophysica Acta 1580: 95-109.

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Crowe A J, Abenes, M., Plant, A., **Moloney, M.M**. (2000) The seed-specific transactivator, ABI3, induces oleosin gene expression. Plant Science 151:171-181.

Deckers H, **Moloney MM** and Baum A (1999) The case for recombinant production of pharmaceutical proteins in plants. Annual Reports in Medicinal Chemistry 34: 237-245

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Saborio F, Moloney MM, Tung P and Thorpe TA (1999) Root induction in *Pinus ayacahuite* by co-culture with *Agrobacterium tumefaciens* strains. Tree Physiology 19: 383-389

Ward KA, Holbrook LA, Lamb N, Abrams S, Reid DM and **Moloney MM** (1999) Structural requirements for biologically active jasmonates: Induction of protease inhibitors and seed specific proteins. Plant Growth Regulation 27: 49-56

Chaudhary S, Parmenter DL and **Moloney MM** (1998) Transgenic *Brassica carinata* as a vehicle for the production of recombinant proteins in seeds. Plant Cell Reports 17: 195-200

Kathiresan A, Nagarathna KC, **Moloney MM**, Reid DM and Chinnappa CC (1998) Differential regulation of 1-aminocyclopropane-1-carboxylate synthase gene family and its role in phenotypic plasticity in *Stellaria longipes*. Plant Molecular Biology 36: 265-274

Kermouni A, Mahmoud SS, Wang S, **Moloney MM** and Habibi HR (1998) Cloning a full-length insulin-like growth Factor-I complementary DNA in the goldfish liver and ovary and development of quantitative PCR method for its measurement. Gen Comp Endocrin 111: 51-60

Layzell DB, Brisson N, Devine MD, **Moloney MM**, Taylor GJ, Timmer V, Yada RY and Wood K (1998) Plant biology and food science in Canada: a vision for the future. Can J Bot. 76: 355-364

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Liu J-H, Selinger LB, Cheng K-J, Beauchemin KA and **Moloney MM** (1997) Plant seed oil-bodies as an immobilization matrix for a recombinant xylanase from the rumen fungus *Neocallimastix patriciarum.* Molecular Breeding 3:463-470.

Markley NA, Young D, Laquel P, Castroviejo M and **Moloney MM** (1997) Molecular genetic and biochemical analysis of *B. napus* PCNA function. Plant Molecular Biology 34: 693-700

Hays DB, Rose P, Abrams SR and **Moloney MM** (1996) Biological activity of optically pure C-1 altered abscisic acid analogs in *Brassica napus* microspore embryos. J Plant Growth Regul 15: 5-11

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Zhang X-H, **Moloney MM** and Chinnappa CC (1996) Analysis of an ABA- and osmotic stress inducible dehydrin from *Stellaria longipes.* Journal of Plant Physiology 149: 617-622

Parmenter DL, Boothe JG, van Rooijen GJH, Yeung EC and **Moloney MM** (1995) Production of biologically active hirudin in plant seeds using oleosin partitioning. Plant Mol Biol 29: 1167-1180

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Refereed Reviews or Book Chapters:

Moloney, MM (2002) Oleosin Partitioning Technology for Production of Recombinant Proteins in Oil Seeds **In** *Handbook of Industrial Cell Culture: Mammalian, Microbial, and Plant Cells* Eds. Vinci, Victor A., Parekh, Sarad R. Humana Press p. 279 – 298.

Moloney, MM (2002) Plant Molecular Farming: Using Oleosin Partitioning Technology in Oilseeds **In** *Plants as Factories for Protein Production* Eds. Hood, E., Howard, J. Kluwer Academic Publishers, Dordrecht, The Netherlands p. 55 – 75.

Moloney MM (2000) Molecular farming using seeds as hosts. *In* Seed Technology (Eds Bewley D and Black M) Sheffield Academic Press. pp 226-253

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Patents:

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Deckers, HM, van Rooijen, G, Boothe, J, Goll, J, **Moloney, MM** (2003) Products for Topical Applications Comprising Oil Bodies. United States Patent No. 6,599,513, issued July 29, 2003.

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Peoples, OP, **Moloney, MM**, Patterson, N, Snell, KD (2003) Modification of Fatty Acid Metabolism in Plants. United States Patent No. 6,586,658, issued July 1, 2003.

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Moloney, MM, Boothe, J, van Rooijen, G. (2003) Oil Bodies and Associated Protein as Affinity Matrices. Publication No. US 2003/0096320, published May 22, 2003.

Szarka, S, **Moloney, MM** (2003) Method for production of multimeric immunoglobulins and related compositions. Publication No. US 2003/0093832, published May 15, 2003.

Moloney, MM, Boothe, J, van Rooijen, G. (2003) Oil Bodies and Associated Protein as Affinity Matrices. Publication No. US 2003/0059910, published Mar. 27, 2003.

Moloney, MM, Boothe,J., van Rooijen, G. (2003) Oil Bodies and Associated Protein as Affinity Matrices. United States Patent No. 6,509,453, issued January 21, 2003.

Deckers, HM, van Rooijen, G, Goll, J., **Moloney, MM** (2002) Products for topical applications comprising oil bodies. United States Patent No. 6,372,234, issued April 16, 2002.

Deckers HM, van Rooijen G, Boothe J, Goll J, Mahmoud S, **Moloney MM** (2001) Uses of Oil Bodies. United States Patent No. 6,210,742, issued April 3, 2001.

Habibi H, **Moloney MM** (2001) Expression of Somatotropin in Plant Seeds. United States Patent No. 6,288,304, issued September 11, 2001.

Deckers HM, van Rooijen G, Boothe J, Goll J, and **Moloney MM** (2001) Oil Body Based Personal Care Products. United States Patent No. 6,183,762 B1, issued Feb. 6, 2001.

Deckers HM, van Rooijen G, Boothe J, Goll J, Mahmoud S and **Moloney MM** (2000) Uses of Oil Bodies. United States Patent No. 6,146,645, issued Nov. 14, 2000.

Cheng K-J, Selinger LB, Liu J-H, Hu Y, Forsberg CW and **Moloney MM** (2000) Xylanase obtained from an anaerobic fungus. United States Patent No. 6,137,032, issued Oct. 24, 2000

Cheng K-J, Selinger LB, Liu J-H, Hu Y, Forsberg CW and **Moloney MM** (1999) Xylanase obtained from an anaerobic fungus. United States Patent No. 5,948,667, issued Sept. 7, 1999.

Moloney MM, van Rooijen GJH, and Boothe J (1999) Oil bodies and associated proteins as affinity matrices. United States Patent No. 5,856,452, issued Jan. 5, 1999

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Moloney MM, Alcantara J and van Rooijen GJH (1998) Method for cleavage of fusion proteins. PCT Application WO 98/49326. Published 11/98

Moloney, MM (1998) Transformation and foreign gene expression in *Brassica spp.* United States Patent No. 5,750,871, issued May 12, 1998.

Moloney MM, van Rooijen GJH, and Boothe J (1998) Oil bodies and associated proteins as affinity matrices. PCT Application WO 98/27115. Published 6/98

Moloney MM, Boothe J, and van Rooijen GJH (1996) Oil bodies and associated proteins as affinity matrices. United States Patent No. 5,856,452, issued Jan. 5/99

Moloney, MM (1995) Transformation and foreign gene expression in *Brassica spp.* United States Patent No. 5,463,174, issued Oct. 31, 1995.

Moloney, MM (1993) Transformation and foreign gene expression in *Brassica spp.* United States Patent No. 5,188,958, issued Feb. 23, 1993.

Moloney MM (1992) Oil body protein cis elements as regulatory signals for seed specific expression. This patent describes the cis-acting sequences of oleosin genes which may be used to drive seed specific expression in transgenic plants. United States Patent No. 5,792,922, issued November 8, 1998

Moloney MM (1991) Oil body proteins as carriers of high value proteins. This patent describes a novel procedure for the production of high value peptides such as pharmaceuticals, enzymes, peptide hormones and adhesives in oilseeds. The method provides for a unique purification process which renders economical the production of such proteins in plant seeds. United States Patent No. 5,650,554, issued Jul 22/97

Hilliard J, **Moloney MM** (1989) Probe for electrofusion, electroporation or like procedure II. United States Patent No. 4,882,281, issued Nov. 21, 1989. This patent describes a novel probe with multiple electrodes for the performance of electroporation / electrofusion experiments. Permits the efficient transformation of bacterial or plant cells using a voltage generator of less than 600V.

Moloney, MM and Hilliard, J (1986) A probe for electrofusion, electroporation or like procedure. US Patent No. 4,695,547. This patent describes a novel probe and set-up for the performance of electroporation/electrofusion experiments. The patent has been licensed by Hoefer Scientific of San Fransisco CA and is the basis of their `Progenitor' series of electroporation devices.

Moloney, MM (1986) Transformation of *Brassica spp* using Agrobacterium vectors. Applied U.S. Patent Office, May 1986. European claims accepted May 1987 #868,640. This patent describes a route to the production of transgenic *Brassica* plants using Agrobacterium vectors. Claims involve several target tissues. Issued March/92.

Impact and Contributions:

Evidence for the impact of my work in plant molecular biology and biotechnology can be found in several examples. I served for 3 years (1995-1999) as Editor of The Plant Journal, the second most cited journal in plant biology. I am a member of the advisory board at the Plant Biotechnology Institute in Saskatoon. I have several consulting relationships with both government departments and private industry. I act as a consultant for Dow AgroSciences Canada and frequently for the Provincial and Federal Government. I have served on NSERC (Strategic 1992-1995) and Alberta Government grant selection committees. I am an invited speaker to many international conferences including the TIGR International Genomics Conference, 2000, those of the International Society for Plant Molecular Biology (Amsterdam, 1994; Singapore, 1997), International Society for Fats and Oils Research (The Hague, 1995) and The Biochemical Society (Bristol, U.K., 1996). I give 7-10 invited seminars per year at Canadian, European or U.S. Universities. I was also the Chair and Co-organizer of the International Society for Plant Molecular Biology Congress in Quebec City, June, 2000.

The award of an NSERC Industrial Research Chair to me in 1995, underlines the interest and commitment of industry to our work and attests to our desire to convert, wherever possible, basic discovery research into useful technology. I was the winner of the Alberta Science and Technology foundation (ASTech) award for "outstanding leadership in Alberta technology" in October of 1997. In 2002, I was appointed to the NSERC Council the governing body of the Natural Sciences and Engineering Research Council of Canada. I am the chair of the NSERC Committee on Research Partnerships which accounts for approximately 35% of NSERC's annual research budget and I am a member of the NSERC Executive Committee.

I am the founder and Chief Scientific Officer of SemBioSys Genetics Inc. a biotechnology company based in Calgary, Alberta. SemBioSys, founded in 1994, employs approximately 45 people and is one of the largest Canadian plant biotechnology companies. SemBioSys Genetics Inc. is a world leader in the expression and

manufacture of recombinant proteins for medicinal and industrial applications, using oilseed plants as the host for their production.

Significant Research Contributions:

Subcellular targeting and topology of lipophilic proteins

Abell, B.M., High, S., Moloney, M.M. (2002) Membrane Protein Topology of Oleosin is Constrained by its Long Hydrophobic Domain. Journal of Biological Chemistry: in press. This paper provides a fundamental analysis to targeting and topology of oleosins as examples of the most lipophilic proteins in nature. From this work it is clear that the length of a hydrophobic stretch is more important than its actual sequence in determining topology on a membrane. Furthermore, we demonstrated that novel signal-anchoring sequences can be derived from oleosins to display proteins on the cytoplasmic side of the ER.

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Oleosins represent a unique class of proteins throughout nature. They have the longest hydrophobic stretches of any protein studied to date. They undergo targeting to oil-bodies through a co-translational process and yet they do not enter the secretion pathway. They associate with a translocation pore on the ER, but do not undergo cleavage of a signal sequence. Our work has provided the basis for understanding which parts of the oleosin are essential to correct subcellular trafficking and how the topology of the protein is maintained on oil-bodies. This work had broader implications for all lipophilic proteins including those of non-plant origin.

Use of oleosins as carriers for recombinant proteins

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Moloney, Maurice M. **US Patent 5,650,554** "Oil Body Proteins as Carriers of High-Value Peptides in Plants" Issued Jul. 22, 1997

As a corollary to our work on oleosin targeting, we discovered that oleosin fusion proteins were also capable of efficient targeting to oil-bodies. This led to the hypothesis that recombinant proteins could be attached to oil-bodies in transgenic plants and then separated from other cellular contents based on floatation centrifugation. This liquid-liquid separation could be performed inexpensively and result in a versatile platform for producing recombinant proteins in seeds. In the above-cited papers this hypothesis was validated and several examples of recombinant proteins have been produced this way.

The technology has been patented and now comprises several patent families worldwide. These patents were used to form SemBioSys Genetics Inc., a Calgary-based biotechnology company currently employing 35 staff.

Regulation of seed-specific gene expression

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Moloney, Maurice M. **US Patent 5,792,922** "Oil-Body Proteins Cis-Elements as Regulatory Signals" Issued Aug. 11, 1998.

My laboratory has performed a substantial amount of work on seed-specific gene regulation. We have been particularly interested in the modulation of transcriptional activity by plant hormones especially abscisic acid. My lab has performed the characterization of oleosin gene promoters and their cis-elements and their interaction with key

transcription factors such as ABI3. This work has resulted in greater insights into the factors needed for high level seed-specific expression, and in the isolation, use and patenting of a broad family of seed-specific promoters with uses in modification of lipid, starch or protein deposition in developing seeds.

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PEPTIDE SUBSTRATES FOR CHYMOSIN (RENNIN)

KINETIC STUDIES WITH PEPTIDES OF DIFFERENT CHAIN LENGTH INCLUDING PARTS OF THE SEQUENCE 101-112 OF BOVINE κ-CASEIN

S. VISSER [a], P.J. VAN ROOIJEN [a], CECILE SCHATTENKERK [b] and K.E.T. KERLING [b]

[a] *Netherlands Institute for Dairy Research (NIZO), Ede and* [b] *Gorlaeus Laboratories Department of Organic Chemistry, University of Leiden, Leiden (The Netherlands)*

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Summary

Kinetic parameters have been determined for the reaction between milk-clotting chymosin (EC 3.4.23.4) and a series of peptides (or their methyl esters) including the amino acid sequence around the enzyme-sensitive Phe(105)-Met(106) bond of bovine κ-casein. In particular, the influence of the substrate's chain length on the kinetic parameters has been studied. Evidence is presented that in the model peptides studied the sequence -Ser-Phe-Met-Ala- with a further residue added to either end (in casu Leu(103) or Ile(108)) is necessary to induce any cleavage by the enzyme. When both the Leu(103) and Ile(108) residues form part of the peptide chain, a marked improvement of the substrate properties is observed. It is suggested that prolyl residues on either side of the sensitive peptide bond form additional sites for secondary enzyme-substrate interactions.

Introduction

Chymosin (rennin, EC 3.4.23.4) is a milk-clotting enzyme, the precursor of which is secreted by the fourth stomach of the calf [1]. During the last few decades it has been established that the process of milk clotting is initiated by the specific proteolysis of so-called κ-casein by the enzyme [2]. This initial action on κ-casein is restricted to the cleavage of the peptide bond 105-106 between phenylalanine and methionine [3—5]. To gain information how this specific cleavage is connected with the structure of the substrate, we have undertaken a systematic study of the substrate specificity of chymosin. For this purpose we have utilized a series of synthetic peptides of different chain length includ-

ing parts of the sequence

-Pro-His-Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-Lys-Lys-
101 103 105 106 108 110 112

around the chymosin-labile Phe-Met bond of bovine κ-casein.

In previous communications [6,7] some of us have reported on the substrate properties of a number of such peptides. Conclusions were based on high-voltage paper electrophoretic patterns after prolonged incubation of the peptides with the enzyme. The main features arising from this work were: First, a minimum chain length of five amino acid residues seems to be a prerequisite for cleavage by the enzyme. Second, the sequence Leu(103)-Ser(104) is of importance. When this sequence is reversed, a case studied by Hill [8,9], a sharp decrease in the rate of cleavage is observed. Third, further chain extension at either side of the sensitive bond enhances the capacity of the peptide to function as a substrate for chymosin.

Independently of this work, other groups have also studied the action of chymosin on parts of κ-casein containing the Phe-Met bond in question. Hill [8, 9] concluded from his experiments with short synthetic peptides as well as with photo-oxidized κ-casein [10] that the enzymic action is accelerated by serine and histidine side chains located close to the Phe-Met bond. Polzhofer [11] found a synthetic pentadecapeptide to be split rapidly and calculated a Michaelis constant for this reaction. Furthermore, he concluded that the His (102) residue has an important role, since the hexapeptide His-Leu-Ser-Phe-Met-Ala appeared to be split by chymosin whereas the pentapeptide Leu-Ser-Phe-Met-Ala was found to be resistant to enzymatic cleavage. Evidence for the importance of the hydroxyl group of Ser(104) was presented by Raymond et al. [12,13] working with peptide substrates slightly different from the parent primary structure.

In the present paper kinetic studies are reported in which special attention has been given to the influence of the chain length of the substrate on the kinetics of its reaction with chymosin. This approach permits an investigation of the effect of secondary enzyme-substrate interactions and may lead to some conclusions about the size of the enzyme's active centre [14].

Materials and Methods

Peptides and their derivatives were synthesized and characterized as described by Schattenkerk et al. [15]. The specificity of their cleavage by chymosin was checked by thin-layer chromatography, paper electrophoresis and by N-terminal group analysis using dansyl chloride [16]. Crude chymosin, isolated as an extract ("rennet") from stomachs of newborn calves, was supplied by the "Coöperatieve Stremsel- en Kleurselfabriek" (Leeuwarden, The Netherlands). From this extract chymosin was purified by DEAE-Sephadex chromatography as described by De Koning [3,4]. The milk-clotting activity of the purified enzyme amounted to $6.5 \cdot 10^6$ Soxhlet units [17,18]. As a reference the "Netherland Standard for the determination of (calf-)rennet strength" furnished by the Government Dairy Station (Leiden, The Netherlands), was used.

Enzyme solution. The enzyme was dissolved to a final concentration of 0.418 M in 0.05 M sodium acetate buffer containing 1 M NaCl (pH 5.25) assuming a molecular weight of 30 000 [1,3,4]. Small portions of the enzyme solution, sufficient for a 1-day experiment, were kept frozen until needed in tightly closed 1 ml vials. With this procedure the proteolytic activity remained unaffected for many months.

Substrate solutions. Freeze-dried peptide (5—10 mg for one duplicate experiment) was brought into contact with a suitable volume of 0.05 M sodium acetate buffer (pH 4.7). After centrifugation of non-dissolved material *, the supernatant was utilized as a stock solution, the concentration of which was established as follows. Duplicate samples, to which known amounts of L-norleucine had been added as an internal standard, were made 6 M in HCl and heated at 110°C in small evacuated tubes for 22 h. The contents were then evaporated to dryness in vacuo. The residues were taken up with sodium citrate buffer (pH 2.2) and analyzed with the aid of an automatic amino acid analyzer (JEOL JLC-5AH). The concentration of peptide initially present was calculated by comparison with a standard mixture of amino acids analyzed in the same way.

Duplicate series of 5—8 different concentrations were prepared by diluting samples of the stock solution with 0.05 M sodium acetate (pH 4.7).

Kinetic measurements. Reactions were carried out at 30°C in a 0.05 M sodium acetate buffer, pH 4.7. The enzymatic cleavage was monitored by the automated ninhydrin assay described elsewhere [20]. Each experiment was started with equal volumes of substrate solution (800 μl) in standard size reaction tubes (5.5 × 0.6 cm). The time of mixing the enzyme with the substrate was kept as short as was necessary to ensure complete mixing (5 s). By doing so, we could minimize the effect of a gradually decreasing initial reaction rate which in preliminary experiments had been observed when the reaction mixture was continuously stirred. This effect, the magnitude of which also depended on the total volume stirred, is probably to be attributed to an inactivation of the enzyme at the air-water interface. The same phenomenon was observed when a substrate in which methionine-106 had been replaced by a different residue (e.g. leucine or norleucine), was used. This rules out air oxidation of the substrate's methionine side chain, resulting in decreasing substrate properties (cf. Results), as the main source of this effect.

Evaluation of kinetic parameters. The kinetic parameters, V and K_m, were calculated from the collected data of two independent experiments each carried out with 5—8 substrate concentrations. A BASIC-programmed Hewlett-Packard calculator, model 9830 A, was used routinely to determine slope and intercept from plots of c/v vs c [21] and $1/v$ vs $1/c$ [22]. The programme provides all necessary calculations including a weighted (v^{-4}) least-squares fit to a straight line with standard errors for the computed parameters [23] and the correction for hydrolytic cleavage discussed below.

Correction of parameters for the extent of initial hydrolysis. For the process:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P_1 + P_2,$$

* Addition of an organic solvent, which is sometimes used to enhance the solubility of substrates, has been avoided in this study as this may affect the kinetics of enzymic action [19].

(E = enzyme, S = substrate, and P = product) the rate equation can be written as:

$$v_0 = \frac{Vc_s^0}{K_m + c_s^0}, \quad (1)$$

where v_0 is the initial velocity; c_s^0 the initial substrate concentration; V the maximal velocity (= k_{cat} × total enzyme concentration) and K_m the Michaelis constant (= $(k_{cat} + k_{-1})/k_1$). When the substrate concentration changes to a considerable extent during the time course of measurement (t) the integrated rate equation should be used:

$$Vt = c_p^t + K_m \ln \frac{c_s^0}{c_s^0 - c_p^t} \quad (2)$$

where c_p^t stands for the concentration of product formed. After expanding the logarithm and rearrangement, one obtains

$$\frac{c_p^t}{t} + \frac{K_m\left\{\frac{1}{2}\left(\frac{c_p^t}{c_s^0}\right)^2 + \frac{1}{3}\left(\frac{c_p^t}{c_s^0}\right)^3 + \frac{1}{4}\left(\frac{c_p^t}{c_s^0}\right)^4 + \ldots\right\}}{t\left(\frac{c_s^0 + K_m}{c_s^0}\right)} = \frac{Vc_s^0}{K_m + c_s^0} \quad (3)$$

The ratio c_p^t/c_s^0 is a measure of the extent of hydrolysis. Its value can be established for each substrate concentration [20]. In Eq. 3 the second term on the left-hand side represents the correction of the apparent initial velocity c_p^t/t for substrate depletion. Since this term contains the parameter K_m, we have used an iterative procedure for the evaluation of correct Michaelis parameters. As a starting value of K_m the result from a linear plot with the apparent initial velocities was taken.

Results

Identical kinetic parameters, V and K_m, were obtained by analysing the c_s^0/v vs c_s^0 plot and the $1/v$ vs $1/c_s^0$ plot only after proper weighting for inversion [24].

In Table I the kinetic parameters k_{cat}, K_m and k_{cat}/K_m for a number of peptides are presented together with their standard errors. The experimental conditions are specified in columns 4 and 5. The maximal depletion of substrate during the time used for the determination of initial velocities, is given in column 6. In the last column the number of experimentally determined initial velocities over the concentration range given, is listed. As may be expected, the values of the separate parameters, k_{cat} and K_m, are more reliable when K_m is well within the range of substrate concentrations.

The pentapeptide ester Ser-Phe-Met-Ala-Ile-OMe (I), which previously [6] was found to be split to a reasonable extent during a 24-h incubation period with the enzyme, appeared to be a poor substrate in terms of k_{cat}/K_m as compared with the substrates V—XIII. The same can be said of other peptides with N-terminal serine and chains extended in the C-terminal direction (substrate

TABLE I

KINETIC PARAMETERS OF THE REACTION BETWEEN CHYMOSIN AND SYNTHETIC SUBSTRATES CONTAINING PARTS OF THE κ-CASEIN SEQUENCE

Pro - His - Leu - Ser - Phe - Met - Ala - Ile - Pro - Pro - Lys - Lys -
101 103 105 106 108 110 112

All experiments were carried out at 30°C in 0.05 M sodium acetate buffer (pH 4.7).

Substrate	Number of residues	Substrate concentration (mM)	Enzyme [a] concentration (nM)	Percentage of hydrolysis	k_{cat} [a] (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	n
Ser(104)→Ile(108)OMe	5	0.43—1.72	2960	0.8— 1.2	0.33 ± 0.10 [b]	8.5 ± 2.7 [b]	0.038 ± 0.002	10
Ser(104)→Pro(109)OMe	6	0.20—1.60	1490	0.9— 3.2	1.05 ± 0.45 [b]	9.2 ± 4.0 [b]	0.114 ± 0.007	10
Ser(104)→Pro(110)OMe	7	0.66—1.76	775	2.1— 3.3	1.57 ± 0.82 [b]	6.8 ± 3.6 [b]	0.231 ± 0.022	12
Ser(104)→Lys(111)OH	8	0.30—1.20	1540	4.4— 5.4	0.75 ± 0.15 [b]	3.2 ± 0.6 [b]	0.239 ± 0.013	10
Leu(103)→Ile(108)OMe	6	0.10—0.80	66	8.6—14.6	18.3 ± 0.9	0.85 ± 0.05	21.6 ± 0.7	15
Leu(103)→Pro(109)OMe	7	0.08—0.64 0.10—0.83	38 30	12.2—21.3 9.1—17.3	37.5 ± 1.7 38.7 ± 1.4	0.71 ± 0.04 0.67 ± 0.03	52.8 ± 1.6 57.5 ± 1.7	16 13
Leu(103)→Pro(110)OMe	8	0.09—0.76	19	7.9—18.3	43.3 ± 2.3	0.41 ± 0.03	105.1 ± 6.9	15
Leu(103)→Lys(111)OH	9	0.06—0.50	19	7.4—14.2	33.6 ± 1.2	0.43 ± 0.02	78.3 ± 2.3	13
Leu(103)→Lys(112)OH [c]	10	0.11—0.45 0.06—0.45	43 29	15.9—24.9 10.6—18.0	31.4 ± 1.6 29.0 ± 1.2	0.49 ± 0.03 0.43 ± 0.02	63.7 ± 2.2 66.9 ± 2.1	13 16
Leu(103)→Lys(112)OH [d]	10	0.05—0.37	29	10.9—19.2	25.3 ± 1.5	0.40 ± 0.03	63.0 ± 2.6	7 [f]
Leu(103)→Lys(112)OH [e] [(106)Met(O)]	10	0.09—0.36	143	9.7—12.3	7.1 ± 0.4	0.84 ± 0.05	8.41 ± 0.16	6 [f]
His(102)→Ile(108)OMe	7	0.09—0.65	52	8.8—17.5	16.0 ± 0.8	0.52 ± 0.03	30.8 ± 1.4	12
Pro(101)→Ile(108)OMe [c]	8	0.10—0.83 0.09—0.71	15 14	5.4—13.0 6.2—16.9	34.8 ± 0.5 32.3 ± 0.5	0.37 ± 0.01 0.31 ± 0.01	94.6 ± 2.1 105.9 ± 2.6	15 16

[a] Calculated assuming a molecular weight of 30 000 for the enzyme.
[b] Values of the separate parameters, k_{cat} and K_m, are rather uncertain, since the v vs c_s^0 plot largely showed first-order kinetics.
[c] Results are given of two duplicate experiments done at a time interval of at least one month.
[d] After preliminary treatment with 2-mercaptoethanol.
[e] After preliminary oxidation of the methionyl residue to its sulphoxide according to the method described by Iselin [25].
[f] Data obtained from a single experiment.

II—IV). Addition of a leucyl residue to the N-terminal part of the peptides I—IV caused an increase of the k_{cat}/K_m parameter by more than two orders of magnitude (substrates V—VIII). Extension with a histidyl residue at the N-terminal side of the hexapeptide ester V hardly influenced the k_{cat}; the increase of the k_{cat}/K_m ratio was predominantly brought about by a change of K_m (XII). Further extension in N-terminal direction with a prolyl residue brought about an increase in k_{cat} and a decrease in K_m. The k_{cat} value (32—35 s^{-1}) found for this octapeptide ester XIII is about the same as that of the heptapeptide ester VI obtained by extension with only one (prolyl) residue at the C-terminal side of peptide V. Comparing the kinetic parameters of the substrates V and VI, one sees that for the longer peptide the K_m is somewhat lower while the k_{cat} is doubled. Addition of a second proline to this sequence, leading to the octapeptide ester Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-OMe (VII), resulted in a further decrease of K_m leaving k_{cat} almost unchanged. As soon as the lysine-111 had been joined to the sequence, the k_{cat} value decreased slightly while the K_m remained unaltered (VIII). However, no definite conclusions in terms of electrostatical effects can be drawn from this, since a free carboxylic end group was introduced simultaneously in the peptide chain in place of the methyl ester group, leaving the net peptide charge unaffected. In fact, an extra positive charge was added only by introduction of the second lysyl residue at position 112 (IX). This hardly altered the kinetic parameters as compared with those of the substrate VIII.

Treatment of the peptide IX with 2-mercaptoethanol to eliminate the possible effect of methionine oxidation did not influence the enzyme kinetics (X). Apparently methionine sulphoxide was not present in the peptide preparation in significant amount. This argument is corroborated by the fact that careful oxidation of the methionyl residue to the sulphoxide resulted in a striking fall of the substrate properties as expressed by a decreasing k_{cat} and an increasing K_m (XI).

Discussion

Considering the results listed in Table I, it appears that the leucyl residue at position 103 is of great importance for the rate of hydrolysis of the substrate, as revealed by a sharp increase in k_{cat} after the introduction of this residue. Two factors might be responsible for this effect. First, the extension of the peptide backbone at the "left-hand" side, which, in addition, may lead to a suitable location of the N-terminal charge with respect to a counterion in the enzyme. Second, the increase in hydrophobicity of the peptide by the addition of the leucyl side chain.

From earlier work [7] it follows that the isoleucine-108 also strongly influences the rate of hydrolysis, since the peptide ester Leu-Ser-Phe-Met-Ala-OMe (thus containing Leu(103) but missing Ile(108)) showed equally poor substrate properties as the peptide ester Ser-Phe-Met-Ala-Ile-OMe (I) listed in Table I. Further evidence for an important role of the residue in position 108 was presented by Raymond et al. [12,13] in comparing the peptides Leu-Ser-Phe(NO_2)-Nle-Ala-OMe (k_{cat}/K_m = 0.1 mM^{-1} s^{-1}) and Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe (k_{cat}/K_m = 12.7 mM^{-1} s^{-1}) as substrates for chymosin.

The tetrapeptide esters Leu-Ser-Phe-Met-OMe, Ser-Phe-Met-Ala-OMe and Phe-Met-Ala-Ile-OMe were all found to be completely resistant to cleavage by chymosin [6].

From the above it is apparent that a minimum chain length of five amino acid residues including the sequence -Ser-Phe-Met-Ala- is essential to bring about any cleavage of the Phe-Met bond. A large jump in the substrate quality is effected when both the Leu(103) and the Ile(108) form part of the peptide chain, as is reflected by the marked differences in k_{cat}/K_m between the substrate series I—IV and series V—VIII. The contribution to the rate of proteolysis of substrate groups at some distance from the bond to be split by the enzyme has also been reported for the hydrolysis of peptides by other proteases such as pepsin [26,27], and chymosin-like enzymes from *Mucor miehei* [28] or from *Mucor pusillus* [29].

As is evident from Table I, additional sites of secondary interaction can be located on amino acid side chains more distant from the labile Phe-Met bond. These substrate groups, however, contribute to a much lesser extent to the substrate quality of the peptide than do the leucine-103 and isoleucine-108 discussed above. The kinetic parameters of the substrates V—XIII (except the oxidized peptide XI) all fall within the same range: k_{cat} 16—43 s^{-1} and K_m 0.3—0.9 mM. The most suitable substrates found in the present study were the octapeptide esters VII and XIII ($k_{cat}/K_m \approx 100$ s^{-1} mM^{-1}). These substrates contain, in addition to the important Leu(103) and Ile(108), one or two prolyl residues which evidently further add to the secondary enzyme-substrate interaction. The prolyl residues might also impart some stabilization to a preferential conformation of the substrate molecule.

A discussion about the role of the histidyl and lysyl side chains is hampered by the fact that all our experiments were carried out at the same pH and ionic strength. The pH of 4.7 has been chosen as being the optimum pH for the action of chymosin on small peptide substrates [12]. It cannot be ruled out that the substitution of an additional charged group in a peptide substrate will have a considerable effect on the pH optimum for enzymic cleavage. The conclusion that the introduction of the lysyl residues at the positions 111 and 112 would not greatly affect the substrate quality (cf. Table I) must therefore be considered with care. In addition to this, a second aspect has to be taken into account when considering the function of the side chain of histidine-102. One can conclude from Table I that under the conditions of our studies, coupling of a histidyl residue to the peptide ester V, leading to substrate XII, only influences the K_m of the reaction to a reasonable extent leaving the k_{cat} almost unchanged. This does not imply, however, that in a more extended molecule (e.g. in intact κ-casein) the positive charge of the histidine side chain will not have any effect on the k_{cat} of the enzymic cleavage. In the present case of small peptide esters the function of the protonated imidazole group may have been taken over completely or partly in the hexapeptide ester V by the positively charged N-terminal residue. Definite conclusions as to the function of the histidyl and lysyl side chains in the enzyme-substrate interactions have to await the results of further experimental work.

A marked influence on the kinetic parameters was observed when the methionyl residue of peptide IX had been oxidized to its sulphoxide (XI). This treat-

ment apparently affected the primary interactions between the enzyme and its substrate.

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We are indebted to Professor Dr. E. Havinga (University of Leiden) and to Dr. P.J. de Koning (NIZO) for continuous interest and valuable discussions. We wish to thank Dr. H.J. Vreeman (NIZO) for critical remarks and mathematical guidance.

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ACTIVATION OF FIBROBLAST PROCOLLAGENASE BY MAST CELL PROTEASES

HENNING BIRKEDAL-HANSEN *, CHARLES M. COBB, ROBERT E. TAYLOR and HAROLD M. FULLMER **

Institute of Dental Research, School of Dentistry, University of Alabama in Birmingham, Birmingham, Ala. 35294 (U.S.A.)

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Summary

Proteases capable of activating procollagenase from gingiva and from fibroblast and macrophage monolayer cultures were harvested from homogenates of canine tumor mast cells. The mast cell proteases lysed casein and Azocoll but not native collagen. In low salt concentrations the enzymes existed as high molecular weight complexes, which were dissociated by increasing the salt concentration above 1.0 M (NaCl, KCl). Gel filtration in 1.4 M KCl separated the protease activity into three peaks, all of which activated procollagenase. Two of the enzymes showed substrate specificities (hydrolysis of *p*-tosyl-L-arginine methyl ester and benzoyl-tyrosine ethyl ester) and reactive center reactivities similar to pancreatic trypsin and chymotrypsin. Based on gel filtration, apparent molecular weights of 160 000 (*p*-tosyl-L-arginine methyl ester esterase), 90 000 (main procollagenase activator) and 36 000 benzoyl-tyrosine ethyl ester esterase) were determined. Activation of procollagenase resulted in a 18—20 000 decrease of the molecular weight. The activation was directly related to the amount of activator added within certain limits. Further addition of activator resulted in proteolytic inactivation of collagenase.

Introduction

Collagenases (EC 3.4.24.3) are enzymes capable of cleaving the helical portion of native collagen molecules in a characteristic manner. A series of studies have shown that animal collagenase may exist in a latent form [1—6]. More-

* Present address: Institute for Cariology and Endodontics, Royal Dental College, 160 Jagtvej, DK-2100 Copenhagen O, Denmark.
** Requests for reprints should be addressed to Dr. Harold M. Fullmer at the Institute of Dental Research, University of Alabama in Birmingham, Birmingham, Ala. 35294, U.S.A.

STUDIES ON POLYPEPTIDES. XIII[1]

Relation between structure and capacity to function as rennin substrate.

(Short communication)

BY

C. SCHATTENKERK, I. HOLTKAMP, J. G. M. HESSING, K. E. T. KERLING and E. HAVINGA

(Laboratory of Organic Chemistry, The University, P.O. Box 75, Leiden)

With the aim of exploring the substrate-specificity of the enzyme rennin, which triggers the milk clotting process by specifically splitting the phenylalanine-methionine linkage in κ-casein[2], we synthesized a series of free peptides or their methyl esters containing the Phe-Met bond and increasing parts of the sequence adjacent to this dipeptide at both sides*. The susceptibility of the peptides towards hydrolysis by rennin was tested by incubation at 37° for 24 hours with a solution of rennin** in 0.05 *M*-sodium acetate buffer (pH 4.7).

The hydrolysis of the substrates was checked by paper electrophoresis at pH 1.8; the substrates and their possible splitting products were employed as references.

The enzyme-substrate ratio was 1/1500; the specific activity of the enzyme preparation amounted to 6.62×10^5 units/mg protein. The results represented in Fig. 1, give a clear picture of the capacity of the peptides to function as a substrate for rennin. This capacity was found to increase with increasing length of the peptide chain containing the Phe/Met bond. A high rate of reaction is observed with the hexapeptide Leu-Ser-Phe-Met-Ala-Ile; shortening the chain from either side diminishes the rate of reaction. We compared the peptides containing the sequence Ser-Leu-Phe-Met-Ala[3] with the sequence Leu-Ser-Phe-Met-Ala[2]. Under identical conditions the latter proved to be the better substrate.

It is intended to collect more detailed information about the features of the peptides essential to functioning as substrates for rennin, by enzyme-kinetic studies, varying a) the reaction time and b) enzyme-substrate ratio.

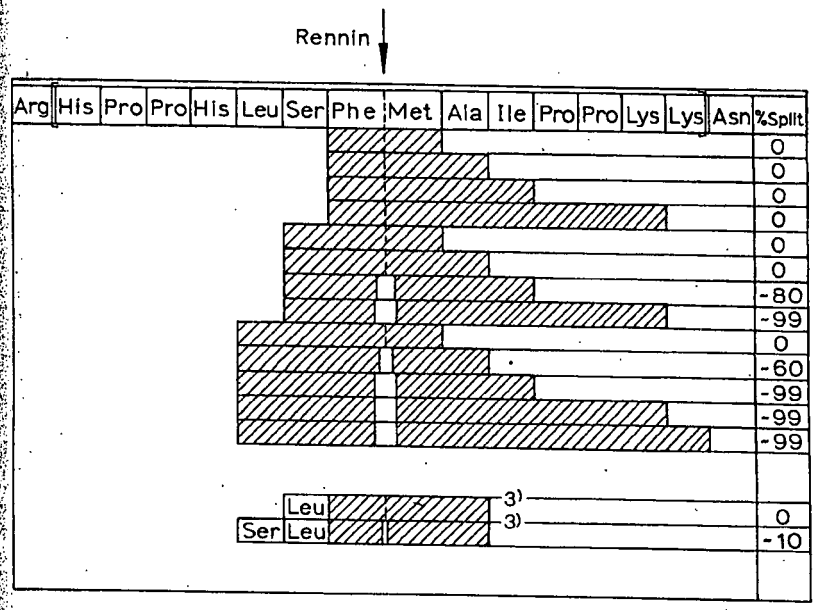

Fig. 1. Action of rennin on synthetic peptides containing the amino acid sequence around the Phe-Met bond of κ-casein.

* The syntheses and the physical properties of the peptides, represented in Fig. 1, will be dealt with in a forthcoming paper. Generally, for the enzymic experiments the methyl esters were used on account of their satisfactory solubilities. For the peptides containing lysine this proved not necessary, the solubilities of the free peptides being sufficiently high.

** Rennin preparations were kindly put at our disposal by Dr. *P. J. de Koning* of the N.I.Z.O. (Netherland Institute of Dairy Research), whom we wish to thank for his cooperation and interest.

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A NOTE ON THE ROSENMUND REDUCTION OF ACID CHLORIDES

(Short Communication)

BY

J. A. PETERS and H. VAN BEKKUM

(Laboratory of Organic Chemistry, Delft University of Technology, Julianalaan 136, Delft, The Netherlands)

According to a recent announcement[1] acid chlorides can be hydrogenated to aldehydes over palladium at ambient temperature and at a pressure of about 3 atm, when fused sodium acetate is added to the solvent (xylene). This prompts us to report our results in the field of acid chloride reduction.

So far, the procedure commonly used in the catalytic reduction of acid chlorides was the original method of *Rosenmund*[2,3] which involves boiling toluene or xylene as the solvent and sulfur-regulated palladium on barium sulfate as the catalyst. The more convenient method of *Sakurai* and *Tanabe*[4] using *N,N*-dimethylaniline as hydrogen chloride acceptor and acetone as solvent has received almost no attention. With this modification acid chlorides can be reduced on palladium at room temperature and at atmospheric pressure. However, we observed that *N,N*-dimethylaniline itself is hydrogenated under these conditions which obscures the end-point of the reduction.

We have developed a modified *Sakurai-Tanabe* procedure for the synthesis of aliphatic and alicyclic aldehydes using palladium on carbon as the catalyst, ethyldiisopropylamine as the hydrogen chloride acceptor and acetone as the solvent. Ethyldiisopropylamine has, like *N,N*-dimethylaniline, the advantage of forming an acetone soluble hydrochloride, so the reaction mixture remains homogeneous. The present formulation enabled smooth reduction of aliphatic and alicyclic acid chlorides at room

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Antibodies produced in plants

A. Hiatt

Transgenic plant systems for the expression of mammalian antibodies offer opportunities for the study of plant metabolism and development. Agricultural production could provide virtually unlimited quantities of any antibody.

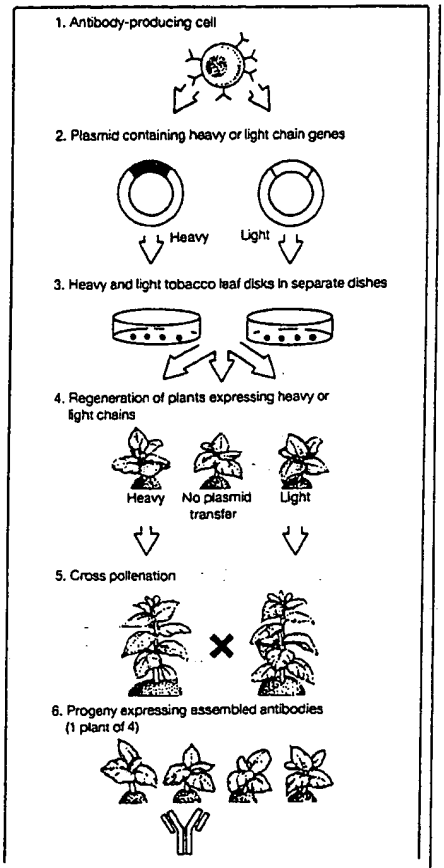
HETEROLOGOUS systems for the expression of mammalian antibodies will undoubtedly contribute a great deal towards our ability to isolate and manipulate immunoglobulins[1]. The latest heterologous host system for antibody synthesis is plants. Techniques to generate transgenic plants have been perfected to the point where a foreign protein can be targeted to an organ of choice as well as to subcellular compartments.

Production of antibodies by plant cells offers a variety of new possibilities for basic research in plant biology as well as for large-scale production of antibodies for use as therapeutic, diagnostic or affinity reagents. The unparalleled capacity and flexibility of agricultural production suggests that antibodies derived from plants may be significantly less expensive than antibodies from any other source. Moreover, antibodies in plants may become useful reagents for manipulating agronomic traits and possibly for ameliorating symptoms of pathogenic infections, as well as for isolating and processing environmental contaminants or industrial by-products.

Plant transformation

Successful expression of an antibody in tobacco has recently been reported[2]. A catalytic antibody[3] was chosen to test the ability of the tobacco cell to assemble and process immunoglobulin chains without compromising functionality. cDNAs encoding heavy and light chains were first inserted into *Agrobacterium tumefaciens*, a soil bacterium that has proven to be very useful for transforming many types of plant cells[4]. The *Agrobacterium* is responsible for transferring the DNA into the plant cell where it is subsequently integrated into the genome. Transformed plant cells are then regenerated to become mature plants[5].

The strategy used for antibody production was to transform tobacco leaf discs and regenerate separate plants expressing either the light or heavy chains (see figure). These plants were then sexually crossed to produce progeny-expressing functional antibody. Although the levels of expression varied widely, greater than one per cent of total protein constituted functional antibody in some plants. There is reason to believe that this level of expression can be augmented by using promoter elements capable of higher levels of transcription[6]. The antibody can easily be purified from homogenized leaves in one affinity purification step. The catalytic properties of the tobacco-produced antibody allow a precise evaluation of kinetic parameters such as K_m, K_i and K_{cat}; by these functional criteria, it is identical to the same antibody derived from hybridoma cells. Further characterization (for example, site of synthesis, secretion, glycosylation) will be reported elsewhere.



Production of antibodies in tobacco plants. Primary regenerants transformed with *Agrobacterium* containing heavy or light chain cDNAs are sexually crossed to enable assembly of a functional antibody in resulting progeny.

Of critical importance, is an evaluation of the immunogenicity of plant-derived antibodies in mammals. As plants do not contain sialyl transferase activity[7], the terminal residues of the carbohydrate on the heavy chain will be different from mammals. In all probability, they will consist of xylose, fucose, and/or *N*-acetylglucosamine[7]. The extent to which alterations in carbohydrate composition affect the biodistribution and serum clearance of the antibody remains to be determined.

Agricultural-scale production

Clearly, if antibodies are to be used for therapeutic purposes, techniques for large-scale production have to be developed. The high capacity and flexibility of agricultural production offers several advantages for obtaining antibodies: genetically stable seed stocks of antibody-producing plants can be isolated and stored indefinitely at low cost and the seed stock can be converted into a harvest of any quantity of antibody within one growing season.

Although tobacco has been used as the principle research tool to initiate the study of antibodies in plants, there may be more appropriate plants for production. A variety of common crop plants can be used as the production host. Acreages of perennial forage crops could be generated by clonal propagation or from seed and harvested numerous times in a growing season. The choice of species may depend on the quantity and nature of contaminants encountered during purification. Some candidates are alfalfa, soybean, tomato and potato.

As large-scale production of antibodies is not yet commonplace, appropriate techniques for the purification of hundreds or thousands of grams have yet to be perfected. The cost of agriculturally-produced antibodies is likely to be considerably less than antibodies produced from hybridoma cells or ascites fluid. For example, if antibodies were expressed in soybean and constituted one per cent of total protein in soybean meal, a kilogram of antibody could, hypothetically, be produced for less than $100 (US). This extrapolation is based on current costs for soybean production and does not take into account numerous hidden costs such as the cost of development and propagation of a sufficiently large and genetically characterized seed stock. In addition, the efficiency with which antibodies can be produced in specialized organs such as seeds or fruit is still not known.

Growth regulation

Plant growth and development is controlled by a limited number of low molecular weight hormones such as indoleacetic acid, ethylene, benzylaminopurine and a variety of more complex organic

molecules[8]. Little is known about the biosynthetic pathways or the mechanism of action of these hormones. However, by expression within the plant cell of monoclonal antibodies that recognise these hormones, it may be possible to evaluate developmental and metabolic events that are controlled by their free titre. Ideally, one would want to control the expression of the antibody as well as target expression to different organs or subcellular locations. In this way, activities of the hormone at various developmental stages could be unravelled.

Pathogen resistance

Antibodies against hormones are just one area where expression of an endogenous antibody could aid plant research. Another example is infection of plants by pathogens. Although many fungal, bacterial and viral pathogens have been characterized with respect to the genetics of host-pathogen interactions, very few have been thoroughly investigated at the biochemical level. In some instances, however, pathogen-related proteins or other organic molecules have been shown to be necessary for pathogenesis[9,10].

Expression of an intracellular antibody that binds antigens essential for pathogenesis may ameliorate the symptoms of the infection by reducing the functional titre. The advantage of this strategy is twofold: first, it would not require isolation of genes involved in synthesis of the target antigen (as with anti-sense RNA expression); and second, pools of antigen which may be localized in subcellular compartments can be the specific target, leaving other pools unaffected. Clearly, the success of this approach will depend on a much more detailed understanding of the behaviour of antibodies in plants. Whereas antibodies have been successfully expressed intracellularly in both yeast and mammalian cells[11,12], attempts to assemble immunoglobulin chains in the cytosol of plants have been unsuccessful.

Current efforts are focusing on alternative methods which would by-pass the requirement for assembly of two immunoglobulin chains (for example, single chain antigen-binding constructs)[13]. In addition, attempts to localize an antigen-binding capacity to chloroplast and vacuole are in progress. Once we have a clear picture of the assembly, stability and functionality of targeted immunoglobulins, appropriate strategies for localized antigen binding can be devised.

Biofiltration

One of the key differences between plant cells and those of other organisms is the structure and characteristics of the surrounding cell wall. The mechanical strength and contiguous nature of plant cell walls is largely responsible for the rigidity of the entire plant. The diameter of pores in the cell wall imposes a restriction on the size of molecules that are freely permeable. This exclusion limit lies between 35 and 50 Å and corresponds to a molecular weight of less than 20,000 for a globular protein. Clearly, antibodies are too large to be freely permeable[14]. Consequently, expression of an antibody in a plant cell is equivalent to producing a binding and retention capacity within a semipermeable membrane. Any antigen with a molecular weight of less than 20,000 (for example, environmental pollutants, industrial by-products, pesticides and herbicides) might be collected and retained by a plant expressing an antibody that is functional *in situ*.

At present, research exploring the applications of biofilters is aimed at characterizing the functional properties of the antibody as it resides within the boundaries of the cell wall. Future efforts will be aimed at enhancing the functionality of antibodies in plants to enable catalytic processing of molecules retained within the cell. □

Andrew Hiatt is a Molecular Biologist at the Research Institute of Scripps Clinic, La Jolla, California 92037, USA. For more information, fill in reader service number 100.

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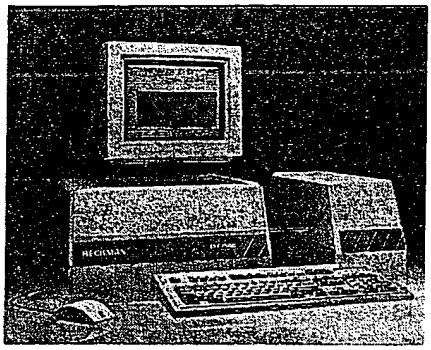
FASEB highlights

The Federation of American Societies for Experimental Biology (FASEB) annual meeting will be held in Washington, DC, next week. A micro-osmotic pump for slow-release drug delivery and a vertical tube gel apparatus will be among the many exhibits.

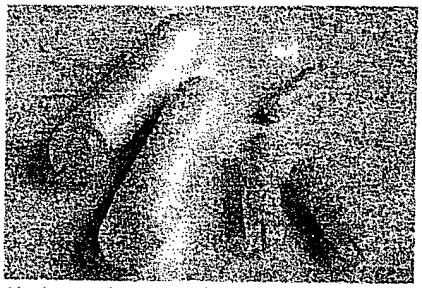
AT FASEB, Beckman Instruments will be launching the programmable DU7500 **diode array UV/visible spectrophotometer** designed for microvolume and ultramicrovolume samples of up to 100 µl (*Reader Service No. 101*). The patented



DU7500: Beckman's next generation of UV/visible spectrophotometers.

full spectrum quantitation applies all the data points in a scan to arrive at accurate and reliable component concentrations, says Beckman. Data are calculated using advanced vector quant maths. RediRead and RediScan modes allow the user to take readings or wavelength scans even when other measurements are in progress. A one-button prompt automatically sets up the new readings or scan, after which the interrupted research can be resumed. The DU7500 simplifies protein analysis by providing pre-selected parameters for Bradford (595 nm), Lowry (high sensitivity: 750 nm; low sensitivity: 500 nm), Biuret (540 nm) and direct UV method (280 nm). Kinetic analyses are run at single or multiple wavelengths: results can be displayed in five plot formats. Prices for the DU7500, which will be in action in booth 1312, range from $15,000–25,000 (US), depending on configuration and choice of accessories.

To meet demands for an **implantable mico-osmotic pump** that can deliver a variety of bioactive compounds to animals weighing less than 10 grams, Alza Corporation has introduced the Alzet Model 1007D (*Reader Service No. 102*). Measuring just 17 mm in length and weighing 350 mg when empty, the Model 1007D provides the controlled administration of



Alza's micro-osmotic pumps provide sustained-release drug delivery.